Naunyn-Schmiedeberg's Arch. Pharmacol. 296, 217–228 (1977)

Naunyn-Schmiedeberg's
Archives of
Pharmacology

Activation of Myocardial β-Adrenoceptors by the Nitrogen-Free Low Affinity Ligand 3′,4′-Dihydroxy-α-Methylpropiophenone (U-0521)*

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Summary. The effect of 3′,4′-dihydroxy-α-methylpropiophenone (U-0521) on the rate of spontaneously contracting cultured rat heart cells and right atria of rats and kittens was investigated. The action of U-0521 on the cellular content of cyclic AMP and on the adenylyl cyclase of heart membrane particles was also studied.

1. U-0521 caused positive chronotropic effects on single cultured heart cells and right atria of the rat. U-0521 was about 10^5 times less potent than (−)-isoprenaline. The maximum effect of U-0521 was smaller than the maximum effect of (−)-isoprenaline. A small positive chronotropic effect of U-0521 was also observed on kitten atria.

2. The β-adrenoceptor blocker (−)-bupranolol antagonized the positive chronotropic effects of U-0521 to the same extent as the effects of (−)-isoprenaline on single cells and atria of the rat. The effects of both U-0521 and (−)-isoprenaline appear therefore mediated through the same β-adrenoceptors. The positive chronotropic effects of U-0521 on kitten atria were also blocked by (−)-bupranolol.

3. Up to 0.1 mM U-0521 did not block the effects of (−)-isoprenaline on rat atria, not even in the presence of corticosterone or hydrocortisone.

4. 1 min incubations with equieffective (increase in cellular beating rate) concentrations of U-0521 (0.1 mM) and (−)-isoprenaline (1 nM) caused a significant increase in the cellular content of cAMP; this effect of both drugs was antagonized by 10 nM (−)-bupranolol.

5. 0.1–3.3 mM U-0521 did not stimulate the adenylyl cyclase of cell-free membrane particles of kitten ventricles. The cyclase was depressed by 10 mM U-0521. 3.3 mM U-0521 caused a 20% decrease of the maximum cyclase-stimulating effect of (−)-isoprenaline and a 1.6-fold increase of its apparent K_m.

6. The results with U-0521 suggest that β-adrenoceptors can be activated by agonists devoid of nitrogen. However, the affinity of U-0521 for the β-adrenoceptor is very low ($K_{U\text{-}0521} \approx 5.5$ mM). The concentration of U-0521 (0.1 mM) causing maximal increases in beating rate of cultured cells probably occupies less than 7% of the available β-adrenoceptors.

Key word: Heart β-adrenoceptor – 3′,4′-Dihydroxy-α-methyl propiophenone (U-0521) – Structure-activity – cAMP – Chronotropism.

INTRODUCTION

3′,4′-Dihydroxy-α-methyl-propiophenone (U-0521) is a substrate of catechol-O-methyltransferase (COMT) (Giles and Miller, 1967a). Under certain experimental conditions the effect of catecholamines on heart (Giles and Miller, 1967b; Kaumann, 1970, 1972; Wöppel and Trendelenburg, 1973) and smooth muscle (Trendelenburg et al., 1971) are potentiated by U-0521, presumably because this drug impairs competitively extraneuronal O-methylation of the amines.

Catecholamines cause positive chronotropic effects on cultured beating heart cells of the rat (Wollenberger, 1964; Boder and Johnson, 1972; Kaumann and Wittmann, 1975). During a preliminary study on rat heart cells we attempted to investigate whether

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* This work was supported by grants of the Deutsche Forschungsgemeinschaft "SFB 30 Cardiologie" and "Biochemie der Morphogenese", and by grant HD-06513 from the National Institutes of Health

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or not U-0521 potentiated the effect of catecholamines. Unexpectedly, U-0521 itself caused an increase of the rate of spontaneously beating cells. The present report deals with some aspects of the positive chronotropic action of U-0521 on heart cells and right atria of the rat. It became apparent that the positive chronotropic effects of U-0521 and (−)-isoprenaline were antagonized competitively to the same extent by the β-adrenoceptor blocker (−)-bupranolol, indicating that the three drugs interacted with similar β-adrenoceptors.

Affinity characteristics of ligands for β-adrenoceptors mediating both positive chronotropic effects and adenylyl cyclase stimulation by catecholamines are similar (Kaumann and Birnbaumer, 1973, 1974a, b), indicating that the same type of β-adrenoceptors mediates at least in part both effects. To gain further insight into a possible interaction of U-0521 with β-adrenoceptors, effects on the cellular content of cyclic AMP and on membrane adenylyl cyclase activity were examined.

METHODS

Monitoring of the Rate of Beating Heart Cells

Cell cultures from hearts of newborn rats were made as described recently by Kaumann and Wittmann (1975). Figure 1 summarizes a system for the recording of the rate of spontaneously beating heart cells. The cell picture observed through the phase contrast microscope was recorded by a TV camera, displayed on the screen of a Saba TV and simultaneously fed into a Grundig video recorder. This technique enabled us to study simultaneously in one experiment the effect of drugs on up to 4 independently beating cells.

Contractions of the cells cause changes in light intensity on the TV screen in the cell area. These changes were recorded by means of a LDR photocell. The photocell BPY 61 Siemens occluded a tunnel of 2 mm ⌀ in the central part of a black plastic block measuring 20 mm ⌀. The block with the recording slit prevented environmental light to reach the photocell. The photocell was stuck to the screen with scotch tape. Cells were adjusted under the photocell on the TV screen in such a position that during intervals between contractions dark cell parts predominated, whereas during contraction brightness developed. To achieve optimal recordings, the phase contrast picture was set so that all cell borders and organelles developed halo-like phenomena.

The output signal of the photocell was amplified by a μA 741 operational amplifier. The photocell was the gain regulator of the circuit. An increase of incoming light caused a proportional increase of the characteristic resistance of the photocell. A variable offset voltage was applied to the amplifier. During darkness the gain of the amplifier was maximum. The offset voltage was adjusted to the brightness of the halo of each individual cell. The amplified output was filtered by a RC combination to climinate the 15625 Hz sweep frequency of the TV, and monitored on a conventional recorder.

Cyclic AMP Test

The samples were prepared by the method of Brown et al. (1971), adapted and modified as follows. The number of cells in a culture vessel was counted using an ocular micrometer of 0.5 mm. Each culture was divided into 9 equal squares. Cells were counted in each of these squares along the micrometer, and in one additional place which was arbitrarily chosen. The 10 counts were averaged and the number of cells per culture calculated.

Batches which had approximately equal numbers of cells for each assay were chosen for comparative experiments. First 5 ml of fresh serum-free medium were pipetted into the culture vessels. The cultures were incubated for about 30 min at 35° C to prevent artifacts due to the changes of the medium. Drugs were injected with Hamilton μl syringes. Drugs were dissolved in 40 μM EDTA.

After injection of the drug the medium was mixed by shaking the vessels 3 times. The cells were incubated 1 min or 5 min with (−)-isoprenaline or U-0521. The medium was frozen for cyclic AMP determination, traces of medium withdrawn with a Pasteur pipette and the whole culture vessel immersed into liquid N_2. After remaining for 10−15 s in liquid N_2 the cells were frozen. Then 1 ml of ice-cold 6% (w/v) trichloroacetic acid was added to the culture vessel. The culture was stored immediately at about −20° C.

Cells were harvested after thawing by thoroughly scratching the bottom of the culture vessel with a bent Pasteur pipette. The vessel was washed twice with 1 ml 6% trichloroacetic acid to collect cell debris. Completeness of the removal of cell debris was checked microscopically during the scratching procedure. Centrifuge tubes containing cell debris were immersed for 1 min into boiling water to obtain a better sediment of cell proteins. After cooling and centrifuging for 10 min at 250 g the sediment was used for protein determination according to the procedure of Lowry et al. (1951). The supernatant was washed 4 times with 2 ml of water-saturated diethyl ether. The washed extract was dried at 30−50° C or lyophilized overnight in a Leybold-Heraeus GT 2 freezedrier. The dried residue was taken up in 0.5 ml acetate-buffer pH 4.0. Cyclic AMP was assayed according to the method of Gilman (1970). A Boehringer radioisotope dilution test with a cyclic AMP-binding protein (catalogue No. 15289) was used. Since traces of medium appeared to decrease slightly the null values, each standard and sample was made by adding 10 μl of culture medium instead of the corresponding 10 μl of redistilled water. With this procedure the calibration curve was linear between 1 and 20 pmoles of cyclic AMP.

Recovery was determined by pipetting 60, 100 and 200 pmoles cyclic AMP into the cell homogenate. 56.8 ± 11.9 ($\bar{x}$ ± S.D.) (N = 3), 90.5 ± 11.0 (N = 6) and 180.0 ± 20.0 (N = 3) pmoles cyclic AMP were recovered, respectively, when the samples were dried at 30−50° C; 56.3 ± 13.1 (N = 3), 97.0 ± 18.0 (N = 3) and 180.0 ± 16.0 (N = 3) pmoles cyclic AMP were recovered, respectively, when the samples were lyophilized.

Preparation of Membrane Particles

Cell-free membrane particles were made from ventricles of reserpine-pretreated (2.5 mg/kg s.c., 20 h) kittens (300−400 g, either sex) essentially as described by Kaumann and Birnbaumer (1974b), except that the second 20 s homogenization step with the Polytron homogenizer was omitted.

Adenylyl Cyclase Assay

Incubation conditions (32.5° C) and materials were those used by Kaumann and Birnbaumer (1974b) except that a) preliminary incubation with compounds to be tested was omitted, because this does not alter equilibria of the drugs with the receptors (Fig. 14 of Kaumann and Birnbaumer, 1974b), and b) the ^{32}P labelled cyclic AMP formed during incubation was isolated by the method of Salomon et al. (1974). Since preparation of stock solutions of 50 mM U-0521 used in the adenylyl cyclase assay required the presence of 0.3% ethanol (the drug was first dissolved in 90%

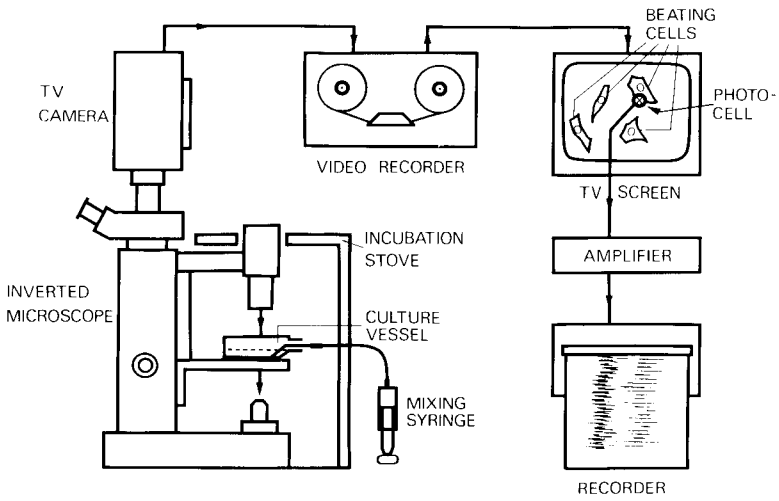

Fig. 1
Experimental setup for the measurement of the rate of several spontaneously beating cells in a single culture. For explanation see text

ethanol and then diluted to 50 mM with distilled water), and since addition of 10 mM U-0521 to the cyclase assay (at the highest concentration tested here) resulted in a final ethanol concentration of 0.06%, all cyclase assays reported here were carried out in the presence of 0.06% ethanol, regardless of whether or not U-0521 was being added.

Isolated Atria

Tissues of female rats (150–250 g) or kittens of either sex (350–1100 g) were used at 32.5°C. The rats were sacrificed by a blow on the head. Kittens were anaesthesized with chloroform. The hearts were rapidly removed and washed free from blood with physiological salt solution. The solution contained (mM): Na^+, 140; K^+, 5; Ca^{2+}, 4.5; Mg^{2+}, 1; Cl^-, 89.5; SO_4^{2-}, 1; HCO_3^-, 29; HPO_4^{2-}, 1; fumarate, 10; pyruvate, 5; L-glutamate, 5; glucose, 10; ethylenediamine tetraacetic acid disodium salt (EDTA), 0.04. Glass-redistilled water was used. The solution was equilibrated with 95% O_2 and 5% CO_2. The hearts were dissected in oxygenated solution at room temperature. Right atria were set up usually in pairs in a 50 ml bath described by Blinks (1965). The atria were suspended at a resting tension just sufficient for measurable development of tension to avoid increases of spontaneous rate by stretching the sinoatrial node (Blinks, 1956). Atrial beating was recorded as described previously (Kaumann, 1972).

Concentration-Effect Curves

Cumulative curves for (−)-isoprenaline or U-0521 were determined by the sequential addition of drugs to the cell culture or isolated organ bath in amounts that increased the total concentration in steps of about 1/2 log unit. Enough time was allowed for each effect to reach equilibrium. Positive chronotropic effects of (−)-isoprenaline and U-0521 were expressed in beats/min on both, cell cultures and isolated atria. SEM of the mean effects were calculated. Four successive concentration-effect curves to (−)-isoprenaline were reproducible and superimposible in 4 rat atria.

Drugs (kindly furnished by the manufacturers). (−)-Isoprenaline bitartrate dihydrate (Sterling-Winthrop, Renselaer); 3′,4′-dihydroxy-α-methyl-propiophenone (U-0521) (Upjohn, Kalamazoo); [(−)-1-(6′-chloro-3′-methyl-phenoxy)-tert.-butylaminopropane-2-ol)] [(−)-KL 255, (−)-bupranolol] (Schwarz-Sanol, Monheim); hydrocortisone acetate, 3-isobutyl-1-methylxanthine (Mix) (Merck, Darmstadt); corticosterone (Calbiochem, Los Angeles); reserpine phosphate (Ciba, Basle). The stock solution of (−)-isoprenaline (10 mM) was acidified with 0.1 N HCl to a pH of about 3 during the initial dilution and left refrigerated. For each experiment appropriate dilutions of (−)-isoprenaline were made in deionized, redistilled water containing 40 μM EDTA.

The purity of U-0521 was checked by gas-chromatography by Dr. Matthiesen, Dept. of Physiological Chemistry, University of Düsseldorf. The sample of U-0521 behaved as a single substance. The synthesis of U-0521 does not involve the use of an amine, either as a reactant or reagent (Dr. P. W. O'Connell, Upjohn Co., Kalamazoo, private communication). Except for experiments with cyclic AMP assays, U-0521 (10 mM) was dissolved in 0.04 N HCl. Hydrocortisone acetate (10 mM) and corticosterone were dissolved in ethylene glycol monomethyl ether. The latter solvent neither changed the rate of spontaneously beating atria or cells nor influenced the effects of (−)-isoprenaline.

Concentrations of all drugs except reserpine are expressed as molarities in contact with the tissues. The dose of reserpine is expressed as mg of base per kg of body weight and was administered i.p. 22–26 h before the experiment. Except for experiments where membrane particles were prepared, the dose of reserpine used was 10 mg/kg.

Statistics. Different groups were compared by a group *t*-test. Statistical significance ($P < 0.05$) of differences of data from the same cell or tissue were assessed by paired *t*-test. Regressions were adjusted by least squares.

RESULTS

Positive Chronotropic Effect of U-0521 on Rat and Kitten Atria; Antagonism by (−)-Bupranolol

Figure 2 shows that U-0521 increased the rate of spontaneously beating atria of the rat. U-0521 was about 10^5 times less effective than (−)-isoprenaline in this respect. Moreover, the maximum effect of U-0521 was smaller than the maximum effect of (−)-isoprenaline. A positive chronotropic effect was also observed with U-0521 on reserpine-pretreated atria (Fig. 3 upper tracing); this effect was somewhat smaller than on atria from untreated rats. Concentrations of 0.3 mM (Fig. 9) U-0521 and greater caused submaximal increases of beating frequency. The

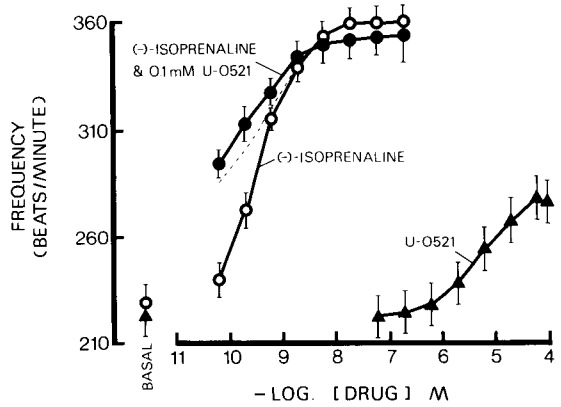

Fig. 2. Comparison of the effects of U-0521 and (−)-isoprenaline on 8 spontaneously beating right atria at 32.5° C. A first concentration-effect curve to (−)-isoprenaline was determined (○). (−)-Isoprenaline was washed out until the stable, basal rate seen prior to the addition of (−)-isoprenaline was recovered. A concentration-effect curve to U-0521, up to 0.1 mM, was then determined (▲). A second concentration-effect curve to (−)-isoprenaline was obtained in the presence of 0.1 mM U-0521 (●). The open circle and closed triangle in the left lower corner are basal rates before the first concentration-effect curve for (−)-isoprenaline and the curve for U-0521, respectively. Symbols and vertical bars are mean rates ± SEM, respectively. It was estimated from interpolation that 0.25 nM (−)-isoprenaline caused the same effect as 0.1 mM U-0521. The broken line was calculated by assuming that (−)-isoprenaline and U-0521 caused additive effects. Since 0.25 nM (−)-isoprenaline was equieffective with 0.1 mM U-0521, responses were calculated (by interpolation) for x nM (determined in the absence of U-0521) plus 0.25 nM (−)-isoprenaline

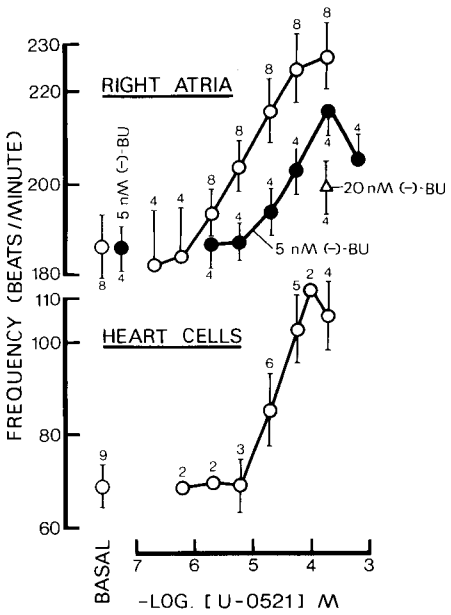

Fig. 3. Comparison of the positive chronotropic effects of U-0521 on right atria and cultured heart cells of the rat. Antagonism by (−)-bupranolol. Cells beating with basal rates between 55 and 85 beats/min were chosen for this group. Right atria were from reserpine-pretreated rats. Cumulative concentration-effect curves to U-0521. After an equilibrium effect with 0.1 mM U-0521 was observed, 20 nM (−)-bupranolol was added to 4 of the right atria and rate recorded 90 min later (△). In another group of atria from reserpine-pretreated rats, a concentration-effect curve for U-0521 was determined 70 min after the administration of 5 nM (−)-bupranolol (●). Figures over symbols indicate number of atria or cells. Symbols and vertical bars are mean rates ± SEM, respectively

positive chronotropic effect of U-0521 was only partially reversible after washing out U-0521. In 4 atria from reserpine-pretreated rats, 0.2 mM U-0521 caused an increase of 44 ± 8 beats/min. After 7 h washing U-0521 out of the bath, these atria were still beating 29 ± 8 beats/min faster than prior to the administration of U-0521. The rate of 4 untreated control atria did not change significantly during a 7 h period.

The high affinity, competitive β-adrenoceptor blocker (−)-bupranolol (Kaumann, 1972; Kaumann and Birnbaumer, 1974b; Kaumann and Wittmann, 1975) (at 50 to 1000 times lower than threshold concentrations for negative chronotropic effects) antagonized the effects of U-0521 (Fig. 3, upper tracing; Fig. 4). The antagonism of the effect of U-0521 by 1 and 5 nM (−)-bupranolol was surmounted by higher concentrations (up to 0.2 mM) of U-0521. However, it was not possible to investigate the competitive nature of the antagonism with concentrations higher than 20 nM (−)-bupranolol because of the depressant effect of U-0521 which would preclude surmountability of the blockade at higher (−)-bupranolol concentrations.

Are the β-adrenoceptors that mediate the positive chronotropic effects of U-0521 similar to those which mediate the effects of (−)-isoprenaline? If the β-adrenoceptors were similar, the effects of both catechols should be antagonized to identical extent by a β-adrenoceptor blocker. To strictly compare concentration-ratios of U-0521 in the presence and absence of (−)-bupranolol with concentration-ratios of (−)-isoprenaline, an experiment was performed with 1 nM and 20 nM (−)-bupranolol to antagonize the effect of (−)-isoprenaline. To explore the (−)-isoprenaline-(−)-bupranolol antagonism over a wide concentration range, 1 μM (−)-bupranolol was also used. The experiment of Figure 5 (top) shows that concentration-effect curves to (−)-isoprenaline in the absence and presence of the three concentrations of (−)-bupranolol were parallel and the blockade was surmounted completely by (−)-isoprenaline. Figure 5 (bottom) shows that the regression of log [concentration-ratio of (−)-isoprenaline-1] vs. −log [(−)-bupranolol] yielded a slope of 0.96, indicating competitive antagonism (Arunlakshana and Schild, 1959). The concentration-ratios of U-0521 and (−)-iso-

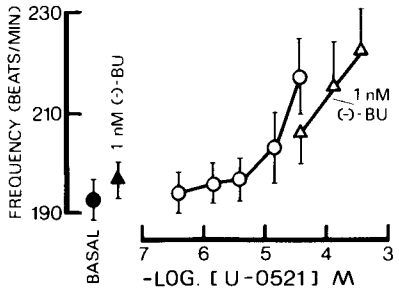

Fig. 4. Surmountable blockade by (−)-bupranolol of the positive chronotropic effects of U-0521 on 4 right atria from reserpine-pretreated rats. A first concentration-effect curve to U-0521 was determined (○). U-0521 was washed out and (−)-bupranolol immediately administered without waiting for complete recovery of the rate prior the addition of U-0521. The ▲ indicates rate after 90 min incubation with 1 nM (−)-bupranolol; a second concentration-effect curve to U-0521 (△) in the presence of (−)-bupranolol was obtained at that time. Symbols and bars are mean rates ± SEM. Concentration-ratios of U-0521 in the presence and absence of (−)-bupranolol were taken at the effect level obtained with 0.14 mM U-0521 in the presence of (−)-bupranolol

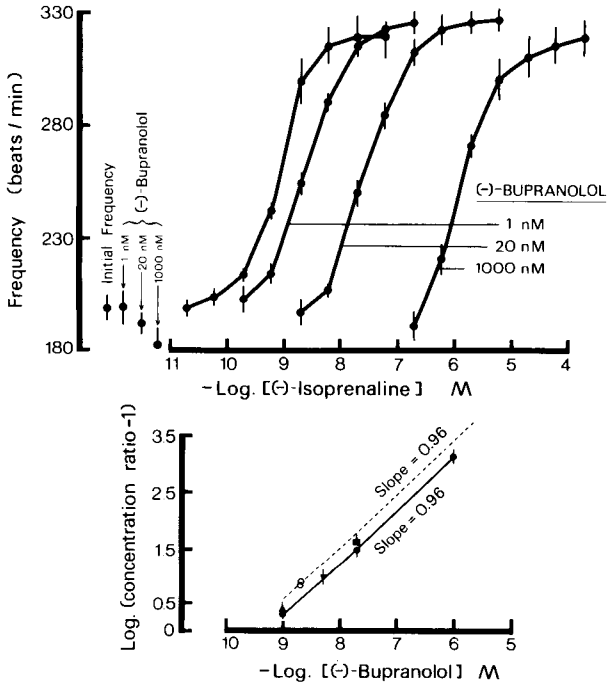

Fig. 5. Antagonism by (−)-bupranolol of the chronotropic effects of (−)-isoprenaline. Top: Right atria from 4 reserpine-pretreated rats. Four successive concentration-effect curves for (−)-isoprenaline were determined in the absence and presence of indicated concentrations of (−)-bupranolol. Bottom: Comparison of double log plots of (concentration-ratio-1) of (−)-isoprenaline [(●) from the upper experiment] or U-0521 (all other symbols) against (−)-bupranolol concentration. Vertical bars are mean ± SEM values. (■,▼ and ▲) are calculated values from the experiments with U-0521 of Figures 3 and 4 respectively. Solid line is a least square regression from values of the upper experiment. Broken line is a least square regression with values from the (−)-isoprenaline vs. (−)-bupranolol antagonism (data from 5 rat heart cells, taken from Kaumann and Wittmann, 1975). Open circles are from the experiment with U-0521 of Figure 7 and an additional experiment

prenaline did not differ significantly with 1 nM or 20 nM (−)-bupranolol (Fig. 5, bottom). The mean concentration ratio of U-0521 in the presence and absence of 5 nM (−)-bupranolol agrees with an interpolated value for a concentration-ratio of (−)-isoprenaline at that antagonist concentration (Fig. 5, bottom). The similarity of concentration-ratios of U-0521 and (−)-isoprenaline in the absence and presence of several concentrations of (−)-bupranolol indicates that all three drugs mediate their effects through the same β-adrenoceptors.

In 6 spontaneously beating kitten atria 0.1 mM U-0521 increased the rate by 10 ± 3 ($\bar{x}$ ± SEM) beats/min ($P < 0.02$). 0.1 mM U-0521 did not increase the rate of 3 additional kitten atria that had been incubated for 60 min with 0.1 μM (−)-bupranolol. The small stimulation of kitten atria by U-0521 agrees with previous observations (Kaumann, 1972, Fig. 8).

The Effects of (−)-Isoprenaline in the Presence of U-0521 in Rat Atria

Figure 2 shows that the concentration-effect curve of (−)-isoprenaline was shifted to the left in the presence of 0.1 mM U-0521. This shift appeared to be greater than expected from additive positive chronotropic effects of U-0521 and (−)-isoprenaline (see legend to Fig. 2). However, the deviation of the observed concentration-effect curve from the expected concentration-effect curve is so small that the statistical significance of this deviation must remain in doubt. The EC_{50} for (−)-isoprenaline in the presence of U-0521 (at the 296 beats/min level) was 0.25 ± 0.06 ($\bar{x}$ ± SEM, $P < 0.01$) log units smaller than the EC_{50} in the absence of U-0521 (at the 315 beats/min level). This small decrease of the EC_{50} of (−)-isoprenaline agrees with a similar decrease of the EC_{50} of (±)-isoprenaline reported by Wöppel and Trendelenburg (1973). Contrary to the marginal influence of U-0521 on the effect of isoprenaline in rat atria, U-0521 causes a 5-fold potentiation of the effects of (−)-isoprenaline in papillary muscles (Kaumann, 1970) and atria (Kaumann, 1972) of kittens.

Positive Chronotropic Effect of U-0521 in Single Rat Heart Cells; Antagonism by (−)-Bupranolol

U-0521 increased the rate of spontaneously beating cultured cells (Figs. 3, 6, 7) in a concentration-dependent manner up to 0.2 mM. Concentrations greater than 0.2 mM caused progressively smaller than maximal increases of rate, presumably because at these high concentrations of U-0521 some toxic action

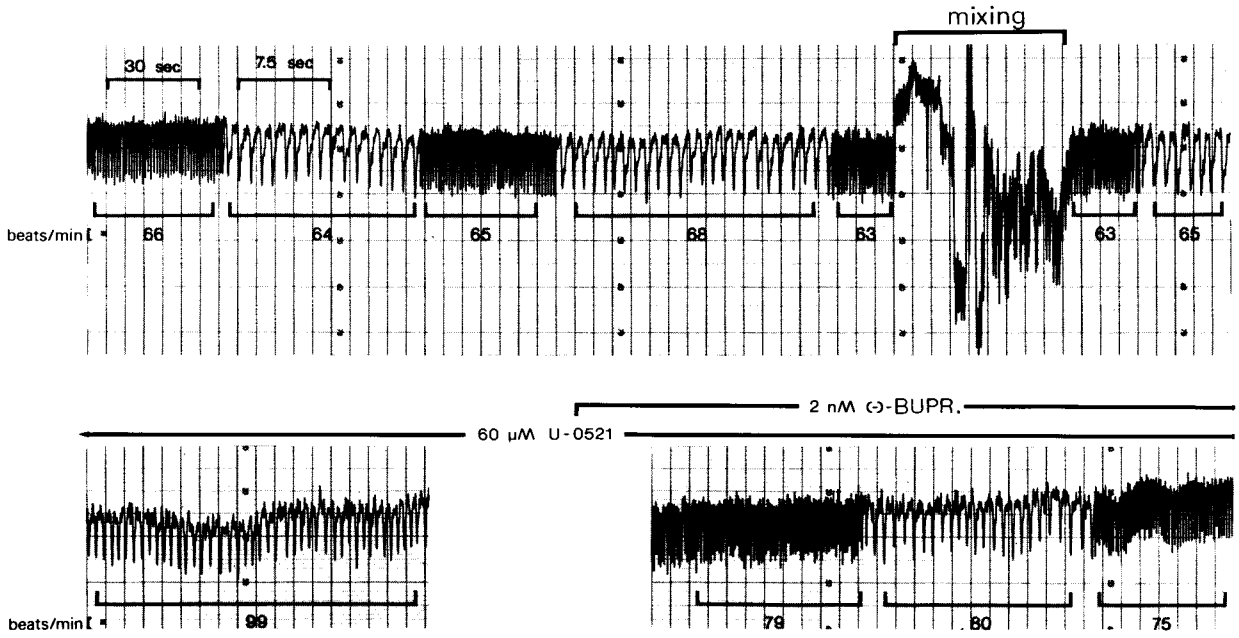

Fig. 6. Antagonism of the positive chronotropic effect of U-0521 by (−)-bupranolol in a cultured rat heart cell. Temperature 35° C. Upper tracings, control rates before U-0521 was administered. Mixing was brought about by withdrawing and reintroducing 1/3 to 1/2 of the medium 5 times into the incubation flask. The left lower tracing was obtained 2 min after the administration of U-0521. (−)-Bupranolol was added to the incubation vessel 3 min before the lower right record was obtained. Mixing artifacts under additions of U-0521 and (−)-bupranolol are not shown. Figures under horizontal bars indicate mean frequencies in beats/min, counted during the time given by the length of the corresponding bar

becomes apparent. The positive chronotropic effect of U-0521 was blocked by (−)-bupranolol (Figs. 6 and 7) but surmounted by higher concentrations of U-0521 (Fig. 7). The concentration-ratios of U-0521 in the presence and absence of 2 nM (−)-bupranolol ($N = 2$) were similar for concentration-ratios expected from the (−)-bupranolol−(−)-isoprenaline antagonism. This is illustrated in Figure 5 (bottom). The broken line was determined from log [concentration-ratios of (−)-isoprenaline-1] vs. log [(−)-bupranolol] from published data (Kaumann and Wittmann, 1975). The log (concentration-ratios of U-0521-1) vs. 2 nM (−)-bupranolol fell close to the broken line of Figure 5. This suggests that U-0521 causes its effects on single, cultured heart cells through the same β-adrenoceptors as does (−)-isoprenaline.

The Influence of Corticosteroids on the Positive Chronotropic Effects of (−)-Isoprenaline. Lack of Antagonism by U-0521 of the Effects of (−)-Isoprenaline in the Presence of Corticosteroids

From the preceding evidence it appears that U-0521 is a partial agonist which causes positive chronotropic effects via activation of β-adrenoceptors. If U-0521 were a classical partial agonist (i.e. causing high receptor occupancy while eliciting effects), one would

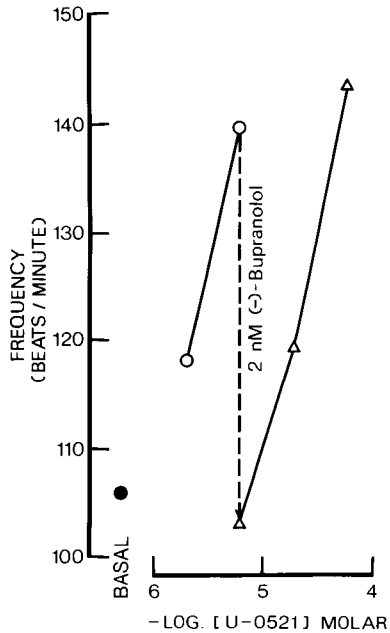

Fig. 7. Surmountable blockade by (−)-bupranolol of the positive chronotropic effects of U-0521 on a 3 days old cultured rat heart cell with pacemaker activity. Temperature 35° C. The effects of two concentrations of U-0521 were tested (○); 2 nM (−)-bupranolol was added in the presence of 60 µM U-0521. (−)-Bupranolol was added 10 min before further U-0521 was administered. △, curve of U-0521 in the presence of (−)-bupranolol. Concentration-ratios of U-0521 in the presence and absence of (−)-bupranolol of this and another experiment are plotted against (−)-bupranolol concentration in Figure 5

expect a shift of the concentration-effect curve of (−)-isoprenaline to the right. However, conceivably U-0521 might simultaneously produce substantial β-adrenoceptor occupancy and prevent O-methylation of (−)-isoprenaline (there is evidence for the latter in the rat heart, Bönisch et al., 1974). The first event would cause a shift of the concentration-effect curve for (−)-isoprenaline to the right, the second to the left. Both events together may cause the sort of result observed in Figure 2. If, in such a situation, extraneuronal uptake of (−)-isoprenaline and therefore its access to COMT were prevented, one would expect to uncover an antagonism between U-0521 and (−)-isoprenaline. For this end we investigated the influence of corticosteroids on the positive chronotropic effect of (−)-isoprenaline in the absence and presence of U-0521.

Hydrocortisone causes potentiation of several effects of (−)-isoprenaline on isolated heart muscle of kittens (Kaumann, 1972). U-0521 in the presence of hydrocortisone produces no further potentiation of the effects of (−)-isoprenaline (Kaumann, 1972). Such experimental results have been interpreted as a blockade by hydrocortisone of the extraneuronal uptake of isoprenaline, thereby preventing the amine from reaching COMT. A somewhat related biochemical situation appears to exist also in the rat heart (Bönisch et al., 1974) although in this species corticosterone rather than hydrocortisone appears to inhibit extraneuronal uptake of amines (Iversen and Salt, 1970). If the effect of (−)-isoprenaline were potentiated by corticosterone in rat atria because the amine is no longer taken up into sites which limit its concentration at the receptors and its access to COMT is prevented, and if it is assumed that U-0521 causes appreciable receptor occupancy, as other β-adrenoceptor partial agonists do (Kaumann, 1973), then U-0521 may perhaps block partially the effects of (−)-isoprenaline in the presence of corticosterone. In 4 atria (not shown) 0.1 mM corticosterone produced only a 0.27 log unit shift of the dose-response curve of (−)-isoprenaline to the left. In the presence of corticosterone the effects of 0.1 mM U-0521 and (−)-isoprenaline were additive. Since hydrocortisone does potentiate effects of (−)-isoprenaline in kitten cardiac tissues (Kaumann, 1972), it was also investigated on rat atria. As in the case of 0.1 mM corticosterone, Figure 8 shows that, while hydrocortisone potentiated the effects of (−)-isoprenaline, it did not cause U-0521 to either potentiate or block the effects of (−)-isoprenaline. The effects of U-0521 and (−)-isoprenaline appeared to be additive in the presence of hydrocortisone, as shown in Figure 8. Assuming that (−)-isoprenaline and U-0521 act competitively on the same receptor, 0.1 mM U-0521 is probably too low

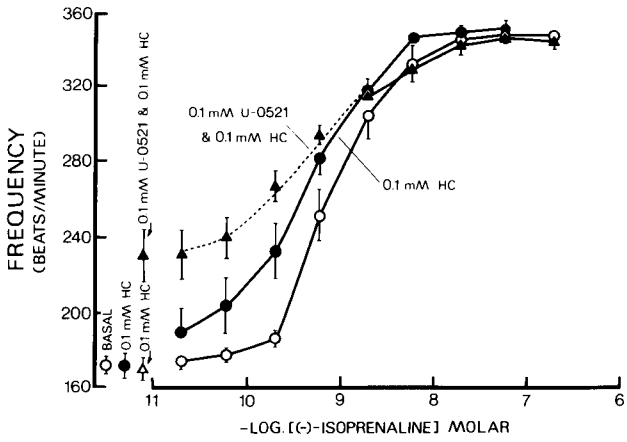

Fig. 8. Potentiation of the effects of (−)-isoprenaline by hydrocortisone. Lack of potentiation of the effects of (−)-isoprenaline by U-0521 in the presence of hydrocortisone (HC). Four atria from reserpine-pretreated rats. A first concentration-effect curve to (−)-isoprenaline was obtained (○). (−)-Isoprenaline was washed out and the tissues exposed to 0.1 mM hydrocortisone thereafter, until the end of the experiment. A second concentration-effect curve to (−)-isoprenaline was then determined 30 min after the addition of hydrocortisone (●). (−)-Isoprenaline was again washed out and 0.1 mM U-0521 was added. 40 min after the addition of U-0521 a third curve to (−)-isoprenaline was determined in the presence of both, U-0521 and hydrocortisone (▲). Symbols and vertical bars are mean rates ± SEM. Open circles, closed circles, and closed triangles on the left side of the picture were basal rates before the 1st, 2nd, and 3rd concentration-effect curve to (−)-isoprenaline was started; open triangles represent basal rates before the addition of U-0521. It was estimated that 0.18 nM (−)-isoprenaline in the presence of hydrocortisone caused the same effect as did 0.1 mM U-0521. Broken lines represent a curve calculated under the assumption that the effects of (−)-isoprenaline and U-0521 (in the presence of hydrocortisone) were additive

a concentration to occupy sufficient receptors to antagonize the effect of (−)-isoprenaline. A 3 times higher concentration of U-0521 was therefore used. However, 0.3 mM U-0521 caused a smaller positive chronotropic effect ($\bar{\Delta}$28 beats/min) than did 0.1 mM U-0521 ($\bar{\Delta}$61 beats/min) (compare Figs. 8 and 9). Furthermore, while depressing the maximum achievable effect of (−)-isoprenaline, it decreased its EC_{50} 2.5-fold (Fig. 9) (mean control EC_{50} taken at the 300 beats/min level; mean EC_{50} in the presence of U-0521 taken at the 290 beats/min level).

Increase of Cellular Cyclic AMP by U-0521 and (−)-Isoprenaline. Relationship to Positive Chronotropic Effects

Results are shown in Table 1 and Figure 10. Equieffective (chronotropic) concentrations of U-0521 (0.1 mM) and (−)-isoprenaline (1 nM) caused significant increases of cellular cyclic AMP when the cells were incubated 1 min (groups A–E of Table 1).

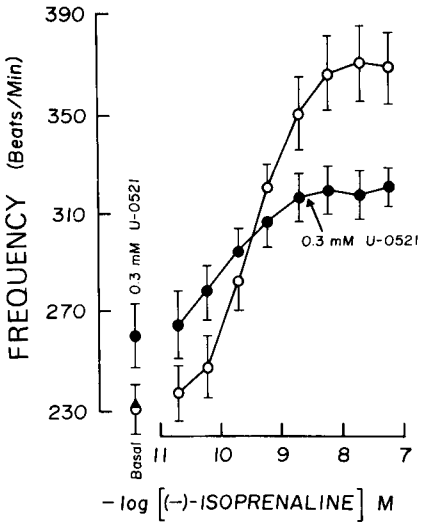

Fig. 9. Positive chronotropic effects of 0.3 mM U-0521 and influence of U-0521 on the effects of (−)-isoprenaline. Mean ± SEM values of 4 atria from reserpine-pretreated rats. 0.1 mM hydrocortisone was in the physiological solution throughout the experiment. A first concentration-effect curve to (−)-isoprenaline was obtained (○). The drug was washed out until a stable rate was observed. 0.3 mM U-0521 was added and a second concentration-effect curve for (−)-isoprenaline (●) determined 40 min thereafter. Symbols and vertical bars are mean rates ± SEM. Open circle, closed circle and closed triangle on the left side of the frame were basal rates before the 1st and 2nd concentration-effect curve to (−)-isoprenaline and the administration of U-0521 respectively

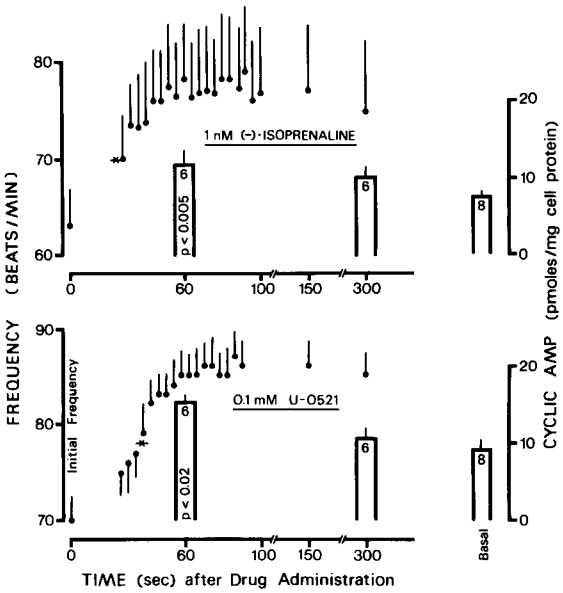

Fig. 10. Similar effects of both 1 nM (−)-isoprenaline ($N = 6$) and 0.1 mM U-0521 ($N = 6$) on rate of spontaneously beating cells (●) and on cellular cyclic AMP (N of cultures in columns, vertical bars SEM). X = half times of onset (mean ± SEM) of chronotropic effects. For further details see text

The magnitude of these increases (expressed as % of initial) of cellular cyclic AMP by 0.1 mM U-0521 and 1 nM (−)-isoprenaline was similar. However, the mentioned concentrations of both drugs did not cause significant increases of cellular cyclic AMP when the cells were incubated 5 min (groups A and B of Table 1, Fig. 10). In contrast to the transient effect of 0.1 mM U-0521 and 1 nM (−)-isoprenaline on cellular cyclic AMP, their respective positive chronotropic effects remained approximately constant from the first to the fifth minute after drug administration (Fig. 10). The half times for onset of the positive chronotropic effect of 0.1 mM U-0521 and 1 nM (−)-isoprenaline were ($\bar{x} \pm$ SEM) 37 ± 3 and 24 ± 2 s, respectively.

It was not sensible to investigate the effect of higher U-0521 concentrations because cells stopped beating a few minutes after the administration of 0.4 mM U-0521.

A low concentration (10 nM) of the high affinity β-adrenoceptor blocker (−)-bupranolol (Kaumann and Wittmann, 1975) prevented the stimulant effect on cellular cyclic AMP by 1 min exposures to both 0.1 mM U-0521 or 1 nM (−)-isoprenaline (groups C and D of Table 1). Pretreatment of cells (from the same batch) with 1 mM of the phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine (Mix) during 15 min caused a significant increase ($P < 0.001$) in their content of cyclic AMP. In such cells, both 0.1 mM U-0521 and 1 nM (−)-isoprenaline significantly increased further the content of cyclic AMP (group E of Table 1).

Cyclic AMP was not detected in the medium, not even 1 min or 5 min after the administration of 0.1 mM U-0521.

Effects of U-0521 on the Adenylyl Cyclase of Membrane Particles

Many β-adrenoceptor ligands are less toxic on membrane particles than on intact cells or tissues (Kaumann and Birnbaumer, 1974b). If U-0521 were less toxic on membrane particles than on tissues and cells, a higher concentration of U-0521 could be used in the former than in the latter two systems, to inquire whether or not it caused significant β-adrenoceptor occupancy. Figure 11 (right graph) shows that up to 3.3 mM U-0521 exhibited only a marginal stimulating

Table 1. The effects of U-0521 and (−)-isoprenaline on the cellular content of cyclic AMP[a]

Condition		min	Cell cyclic AMP pmoles/mg protein $\bar{x} \pm$ SEM	Significance of difference between control and catechol-treated
A Control[d]		1	9.3 ± 1.2 (6)[b]	
0.1 mM U-0521		1	15.5 ± 0.8 (6)	$P < 0.02$[e]
0.1 mM U-0521		5	10.7 ± 1.3 (8)	
B Control[d]		1	7.7 ± 0.42 (8)	
1 nM (−)-isoprenaline		1	12.0 ± 1.27 (6)	$P < 0.005$[e]
1 nM (−)-isoprenaline		5	10.2 ± 1.7 (6)	
C Control[d]		1	6.3 ± 0.3 (3)	
0.1 mM U-0521		1	10.0 ± 1.0 (4)	$P < 0.025$
10 nM (−)-bupranolol 25 min	Control[d]	1	7.0 ± 0.4 (4)	
	0.1 mM U-0521	1	6.8 ± 0.7 (4)	
	1 nM (−)-isoprenaline	1	6.5 ± 0.7 (4)	
D Control[d]		1	7.9 ± 0.6 (8)	
1 nM (−)-isoprenaline		1	12.7 ± 1.6 (6)	$P < 0.01$
10 nM (−)-bupranolol 25 min	Control[d]	1	7.0 ± 0.6 (3)	
	1 nM (−)-isoprenaline	1	6.8 ± 0.6 (3)	
E Control[d]		1	10.7 ± 0.8 (8)	
0.1 mM U-0521		1	16.4 ± 1.4 (8)	$P < 0.005$
1 nM (−)-isoprenaline		1	13.3 ± 0.8 (6)	$P < 0.05$
1 mM Mix[c] 15 min	Control[d]	1	19.9 ± 1.3 (8)	
	0.1 mM U-0521	1	27.9 ± 1.7 (8)	$P < 0.005$
	1 nM (−)-isoprenaline	1	53.0 ± 1.6 (6)	$P < 0.001$

[a] Cultures from the same batch of hearts indicated by capital letters
[b] Number of cultures between parentheses
[c] Mix is 3-isobutyl-1-methylxanthine
[d] 0.4 μM EDTA
[e] Results also shown in Figure 10

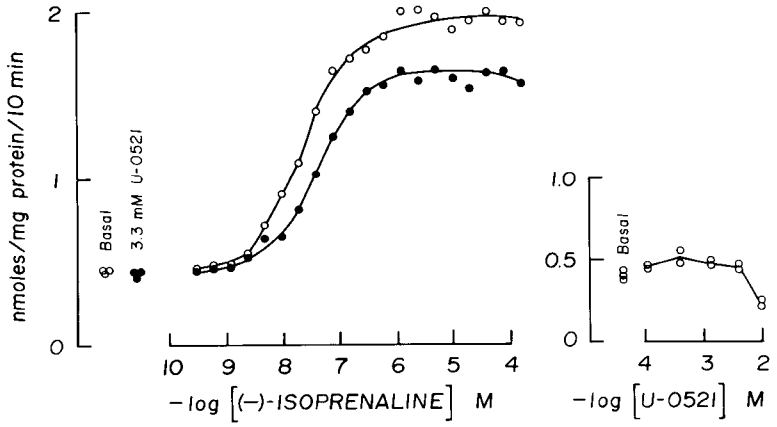

Fig. 11
Effect of U-0521 on the adenylyl cyclase and cyclase-stimulation by (−)-isoprenaline in cell-free membrane particles of kitten ventricle at 32.5° C.
Each symbol represents a separate incubation.
The right picture shows a concentration-effect curve for U-0521.
The left picture shows concentration-effect curves to (−)-isoprenaline in the presence (●) and absence (○) of 3.3 mM U-0521, respectively. Lines through the concentration-effect curves of (−)-isoprenaline were drawn by eye

effect on the cyclase. 10 mM U-0521 depressed slightly cyclase activity. If 3.3 mM U-0521 caused sufficient β-adrenoceptor occupancy it should block competitively the cyclase stimulating effect of (−)-isoprenaline. Figure 11 (left graph) shows that 3.3 mM U-0521 depressed by 20% the maximum effect of (−)-isoprenaline. The apparent K_m of (−)-isoprenaline was increased only 1.6-fold, indicating that U-0521 causes little if any β-adrenoceptor occupancy.

DISCUSSION

The positive inotropic effect of U-0521 has the following characteristics. 1. It persists at least partially

in atria from reserpine-pretreated rats. 2. It is observed in cultured heart cells of the rat. These two pieces of evidence demonstrate that the effect of U-0521 is in great part not due to a release of endogenous noradrenaline, because nerves of atria of reserpine-pretreated rats have their stores of noradrenaline depleted and because cell cultures are devoid of nerves. 3. The competitive β-adrenoceptor blocking agent (−)-bupranolol (1−20 nM) antagonizes the effects of U-0521. The blockade is surmountable by higher concentrations of U-0521. 4. Concentration-ratios of U-0521 and (−)-isoprenaline in the presence and absence of the β-adrenoceptor blocker (−)-bupranolol were similar and were the concentrations-ratios expected from simple competitive antagonism. Support for this mode of drug-receptor interaction comes from the fact that the slope of the regression of log [concentration-ratio of (−)-isoprenaline-1] against log [(−)-bupranolol] was close to unity with data from atria (slope 0.96 present report) and cells (slope 0.96, Kaumann and Wittmann, 1975) as expected from theory (Arunlakshana and Schild, 1959). 5. U-0521 in concentrations of up to 0.3 mM does not antagonize competitively the effects of (−)-isoprenaline, not even under experimental conditions were capture of (−)-isoprenaline by tissues is presumably blocked (corticosterone−Iversen and Salt, 1970) and O-methylation is simultaneously prevented. 0.1 mM of each, hydrocortisone or corticosterone, potentiated slightly the effects of (−)-isoprenaline. U-0521 did neither antagonize nor potentiate the effects of (−)-isoprenaline in the presence of either corticosteroid. However, at this concentration only corticosterone but not hydrocortisone appears to prevent extraneuronal uptake of catecholamines (Iversen and Salt, 1970). Thus, prevention of extraneuronal uptake of catecholamines may not offer an adequate explanation for the slight potentiation by corticosteroids of the effect of (−)-isoprenaline in rat atrium. The lack of antagonism of the positive chronotropic effects of (−)-isoprenaline by U-0521 under various experimental conditions indicates that U-0521 causes very low β-adrenoceptor occupancy while causing its own positive chronotropic effects.

The cellular content of cyclic AMP was increased to similar extent by 1 min but not by 5 min incubations with 1 nM (−)-isoprenaline or 0.1 mM U-0521, while both catechols caused a similar stable increase in beating rate of the cells from the first to the fifth minute after drug administration. The increase of cellular cyclic AMP by either 1 nM (−)-isoprenaline or 0.1 mM U-0521 was prevented by β-adrenoceptor blockade with 10 nM (−)-bupranolol. The characteristic increase of cellular cyclic AMP by U-0521 and (−)-isoprenaline and the blocking effect of (−)-bupranolol are consistent with a β-adrenoceptor-mediated action of both catechols. Independent evidence in favour of U-0521-β-adrenoceptor interaction is the quantitative agreement of the antagonism by (−)-bupranolol of the chronotropic effects of both U-0521 and (−)-isoprenaline. Thus, the positive chronotropic effects of both U-0521 and (−)-isoprenaline appear to be mediated through the same β-adrenoceptors. An observation of Trendelenburg et al. (1971) is qualitatively consistent with this concept. These authors mentioned that U-0521 causes a positive chronotropic effect on rat atria, and that this effect is blocked by the β-adrenoceptor blocker (±)-propranolol.

More than a dozen β-adrenoceptor blockers antagonize competitively the effects of (−)-isoprenaline to a very similar extent in intact tissues and membrane particles of heart tissues (Kaumann and Birnbaumer, 1973, 1974b). However, 0.1 mM U-0521 had no blocking effect in membrane particles (Kaumann and Birnbaumer, 1974b). In the present report 3.3 mM U-0521 decreased the apparent K_m of (−)-isoprenaline for cyclase stimulation only 1.6 times, i.e. by 60% (close to the limit of detection). This slight reduction of the apparent affinity may be partially related to the 20% concomitant decrease of maximum cyclase stimulation by (−)-isoprenaline. Thus, 3.3 mM U-0521 inhibits the effect of (−)-isoprenaline in a partially non-competitive manner. This makes it difficult to estimate accurately whether U-0521 actually occupied any β-adrenoceptors used by (−)-isoprenaline to stimulate the cyclase. Assuming that both, (−)-isoprenaline and U-0521, act through the same receptors, and neglecting the influence of the depressant action of U-0521 on the maximum effect of (−)-isoprenaline, a $K_{U\text{-}0521}$ can be roughly estimated. A K_m ratio of 1.6 for (−)-isoprenaline in the presence and absence of 3.3 mM U-0521 would give a $K_{U\text{-}0521}$ of 5.5 mM (from [U-0521] · $[(K_{m(ISO+U\text{-}0521)}/K_{m(ISO)}) - 1]^{-1}$). Thus, if it is assumed that both, U-0521 and (−)-isoprenaline, cause their positive chronotropic effects through the same β-adrenoceptors, the maximum effective concentration of U-0521 (0.1 mM) does so by occupying less than 7% of the available receptors. This is at variance with β-adrenoceptor partial agonists which always cause positive chronotropic effect with relatively high receptor occupancies (Kaumann, 1973).

As discussed above, it is not clear whether or not U-0521 (3.3 mM) causes appreciable receptor occupancy in membrane particles because of the depression of the maximally stimulating effect of (−)-isoprenaline on adenylyl cyclase. In any event, it could only occupy relatively few of the (−)-isoprenaline receptors and cannot be classified as a conventional partial agonist because such a drug would be expected to cause high receptor occupancy while eliciting low

stimulatory effects. Perhaps U-0521 is a potential full agonist but this cannot be seen experimentally because of its toxic effect that prevents it from producing greater stimulatory effects.

Another unusual feature which makes U-0521 a non-conventional agonist is the fact that its molecule has no nitrogen. This may explain partially its low affinity for the β-adrenoceptors (see also Ariëns, 1964). It demonstrates that the nitrogen is not essential to generate positive chronotropic effects mediated through β-adrenoceptors.

The keto group of U-0521 may stabilize the compound (i.e. decrease its internal energy) through a mesomeric effect with the hydroxyphenyl group. It is therefore likely that the position of this group is coplanar to the ring. This confers to the molecule a greater degree of rigidity than observed in catecholamines which have a β-OH group. This relatively rigid configuration may prevent the keto group from approaching closely enough the site of the β-adrenoceptor which corresponds to the β-OH group of aminic ligands. Since the β-OH group of (−)-isomers of aminic ligands enhances affinity up to 50-fold in several myocardial β-adrenoceptor systems (Kaumann and Birnbaumer, 1973, 1974b), the very low affinity of U-0521 may be in part related to the conformational rigidity and to the lack of the β-OH group.

The catechol group of U-0521 is somehow related to positive chronotropic action, as has been suggested by other authors for catecholamines (see Ariëns, 1964, p. 256). However, since both, dopamine (which lacks the β-OH group) and (+)-noradrenaline, but not U-0521 cause maximal positive chronotropic effects similar to those of the optical isomers of isoprenaline (Blinks, 1964), perhaps the lack of nitrogen prevents U-0521 from causing more pronounced effects. In addition a toxic action of U-0521 may also prevent the right atrium from beating faster.

Ariëns (1964, p. 254) stated that the catechol group contributes especially to the affinity of sympathomimetic drugs for β-adrenoceptors. However, the relatively low affinity of the catechol derivative U-0521 for the β-adrenoceptors makes it unlikely that the catechol determines affinity to any important extent.

Acknowledgements. A.J.K. thanks Prof. Dr. R. Kaufmann for his hospitability while he was a visiting scientist at the University of Düsseldorf. We are grateful to Prof. Dr. J. Moser for advice during this work, to Sibille Holzmann for the artistic work, to Sonja Wörner and Sylvia Berger for technical assistance, to G. Gördes for building the incubation stove, to Dr. U. Matthiesen for the gas chromatography of U-0521.

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Received August 11 / Accepted November 15, 1976